Muscle Fiber Conduction Velocity and Enzyme Histochemistry of Muscle in a Rat Model of Chronic Cerebral Hypoperfusion

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ABSTRACT. A rat model of chronic cerebral hypoperfusion was prepared to clarify the effect of reduced cerebral blood flow on histological and electrophysiological changes in muscle fibers. After the bilateral common carotid arteries of eight-week-old rats were bound with a ligature, the rats were reared for another four weeks or eight weeks. Following measurements of muscle fiber conduction velocity (MFCV) in the soleus muscle, enzyme histochemical assessment of the same muscle was performed using ATPase staining. MFCV and muscle fiber size were compared to those of normal rats.

The mean cross-sectional area of muscle fibers decreased in every fiber type except type 2B after the ligation of bilateral common carotid arteries. Since the rats did not exhibit motor paralysis judging by appearances, the decrease in muscle fiber area was considered to be indicative of disuse muscle atrophy. In addition, the mean MFCV value decreased significantly after the ligation of bilateral common carotid arteries, and there was a significant correlation between MFCV and muscle fiber area. Although both muscle fiber atrophy and decreased MFCV improved in eight weeks after the ligation of bilateral common carotid arteries, conversion of muscle fiber types from type 2 to type 1 progressed more than in normal rats.

Key Words ① **muscle fiber conduction velocity (MFCV)** ② **muscle fiber area**
③ **muscle fiber type distribution** ④ **reduced cerebral blood flow**
⑤ **multiple point stimulation(MPS)**

INTRODUCTION

Muscle fiber conduction velocity (MFCV) is reported to decrease in many diseases in which skeletal muscles are affected, such as muscular dystrophy, polyneuropathy, and denervated muscles[1)~4)]. Nevertheless, there have been few reports on the relationship between MFCV and disuse muscle atrophy in which pathological changes have not been found in their muscle fibers[5),6)]. Disuse muscle atrophy occurs not only as a result of inactivity accompanying treatments of some diseases of the internal organs, but also in non-paralyzed muscles of patients with cerebrovascular dementia. However, paralyzed muscles affected by central nervous system disorders can be presumed to be frequently accompanied by disuse muscle atrophy.

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Cerebrovascular dementia frequently coexists in patients with vast ischemic changes in the cerebral white matter secondary due to a cerebrovascular accident or decrease in cerebral blood flow as a result of cerebrovascular stenosis or occlusion. These conditions cause gait disturbances and decrease in activities of daily living. In previous reports in human studies, MFCV decreased in disuse muscle atrophy[5),6)]. In addition, enzyme histochemistry has shown muscles to be affected by central paralysis resembling disuse muscle atrophy[7)]. A significant correlation has also been shown between MFCV and the cross-sectional area of muscles[6)]. Animal studies using enzyme histochemistry have shown the same tendency in inactivity between MFCV and muscle fiber area[8)]. However, to the best of my knowledge, there have been no studies concerning simultaneous measurements of MFCV and enzyme histochemical assessment of muscle fibers affected by central nervous system disorders. In the present study, a rat model of chronic cerebral hypoperfusion was prepared, and the rats were maintained with reduced cerebral blood flow. The relationship between MFCV and histochemical changes in muscle fibers (muscle fiber type distribution and cross-sectional area) were investigated.

MATERIALS AND METHODS

The aimals used in this study were eight week-old male Wistar rats. For comparison, these rats were divided into a control group of 20 rats and a chronic cerebral hypoperfusion group of 20 rats. For the chronic cerebral hypoperfusion model, an incision was made in the skin of the neck under sevoflurane and nitrous-oxide anesthesia. The bilateral common carotid arteries were bound permanently with a ligature, and the incised skin was sutured completely[9)]. None of the rats displayed motor paralysis in the limbs judging by appearances after this procedure. Each rat was put in a cage W 260 × T 360 × H 200 mm in size, and was allowed to move freely in the cage. No particular exercise stress was given to the rats. The room temperature was kept at a constant 25 ℃, and the light of the room was switched on for 12 hours each day. The animals were allowed free access to a solid diet and water. After surgical procedures, 10 rats were reared for another four weeks (chronic cerebral hypoperfusion group : Ch1 group), and the remaining 10 rats were reared for eight weeks (Ch2 group). In addition to the chronic cerebral hypoperfusion groups, eight-week-old rats without surgical procedures were reared in the same cages : 10 rats for another four weeks (control group: Co1 group) and the remaining 10 rats for eight weeks (Co2 group).

After the scheduled periods, the rats were prepared for MFCV measurements and enzyme histochemistry of the muscles. In a room with a temperature of 25 ℃, pentobarbital sodium (50mg/kg) was administered intraperitoneally to the animals, and an incision was made from the lateral to the posterior aspect of the right femur under total anesthesia. The sciatic nerve and its branches were made sure, and the soleus muscle was exposed carefully so as not to damage nerves or muscles. During exposure of the soleus, the gastrocnemius muscle was resected with great care to avoid any effects of volume conduction generated by the gastrocnemius. After exposure, the right hind limb was immobilized so that the ankle joint was kept at 0° to maintain constant tension in the muscles. The depth of anesthesia was kept at a level sufficient for the animals not to feel pain.

Multiple point stimulation (MPS)[10),11)], a technique that obtains each muscle action potential of a single motor unit (single motor unit potential : S-MUP), was used as a method of nerve stimulation to measure MFCV. The advantages of MPS are that S-MUPs with different latency, amplitude, and duration can be

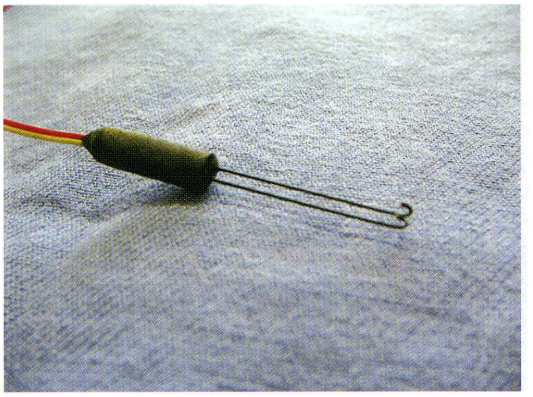


Fig 1. A pair of hook-like electrodes for stimulation.

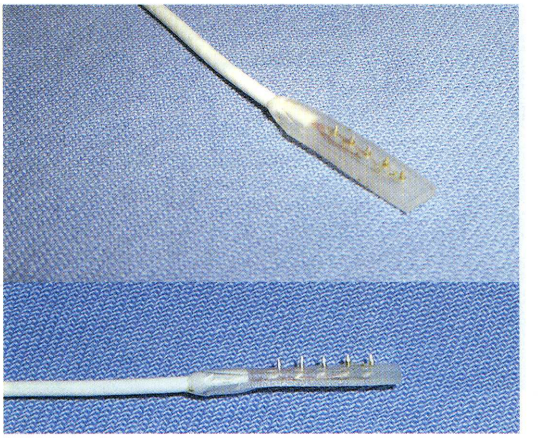


Fig 2. Four-channel surface electrode array.

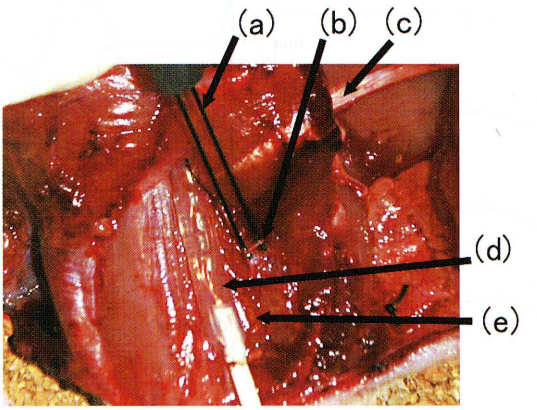


Fig 3. Surgical procedure of the rat hind limb.
(a) a pair of hook-like electrodes for stimulation
(b) Branch of tibial nerve
(c) Sciatic nerve
(d) Four-channel surface electrode array
(e) Soleus muscle

recorded and that MFCVs of various speeds can be obtained[12]. Various sites along the tibial nerve, a branch of the sciatic nerve that innervates the soleus, were stimulated electrically using a pair of hook-like electrodes (Fig. 1). Attention was paid to achieving the minimum stimulation intensity level at which an S-MUP could be recorded. The recording electrode used in the present study was a modified form of the 4-channel surface electrode array (Unique Medical) proposed by Metani[12] (Fig. 2). The surface electrode array had silver disc electrodes 0.2 mm in diameter, arranged on a silicon sheet with thickness of 1 mm; five electrodes were arranged in a row so that each interelectrode distance was 2 mm. The surface electrode array was placed in the direction of muscle fibers of the soleus (Fig. 3). A grounded electrode was placed on the femur on the same side. The Neuropack$\sum$ (Nihon Kohden) was used for electromyography (Fig. 4) and the SEN-3301 (Nihon Kohden) was used as the stimulation (Fig. 5). High and low cut filters were set at 2 KHz and 20 Hz, respectively. The duration of stimulation was 0.1 ms, the stimulation frequency was 1 Hz, and the stimulation intensity was increased gradually by 0.01mA increments. When an S-MUP was detected

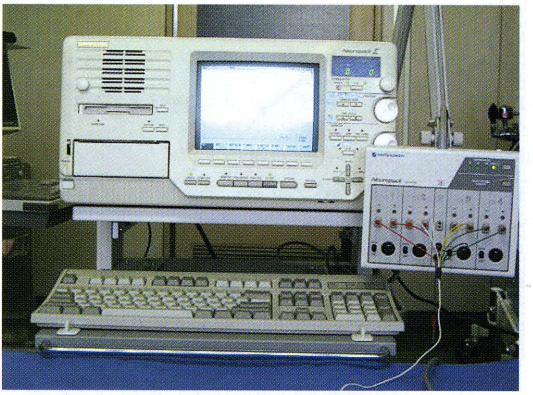


Fig 4. Equipment for electromyography (NeuropackΣ).

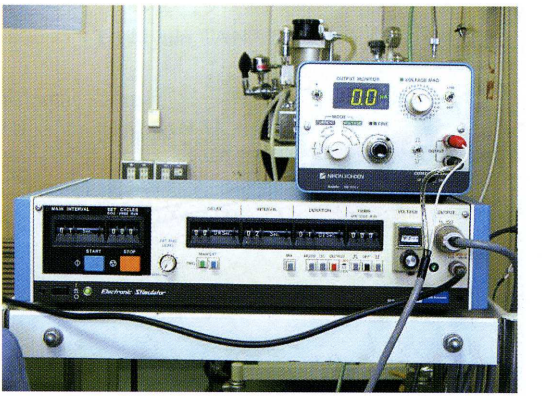


Fig 5. Isolator (top) and stimulator (bottom)

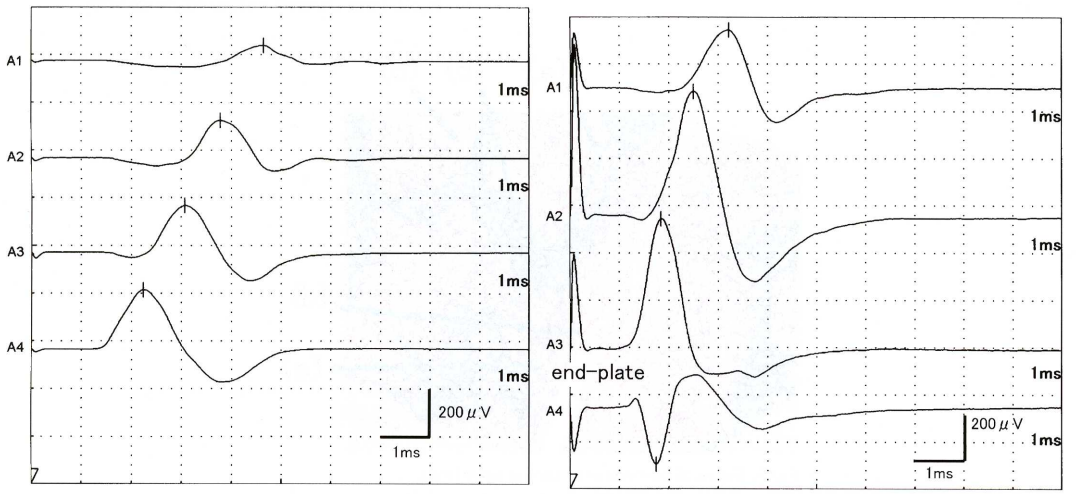
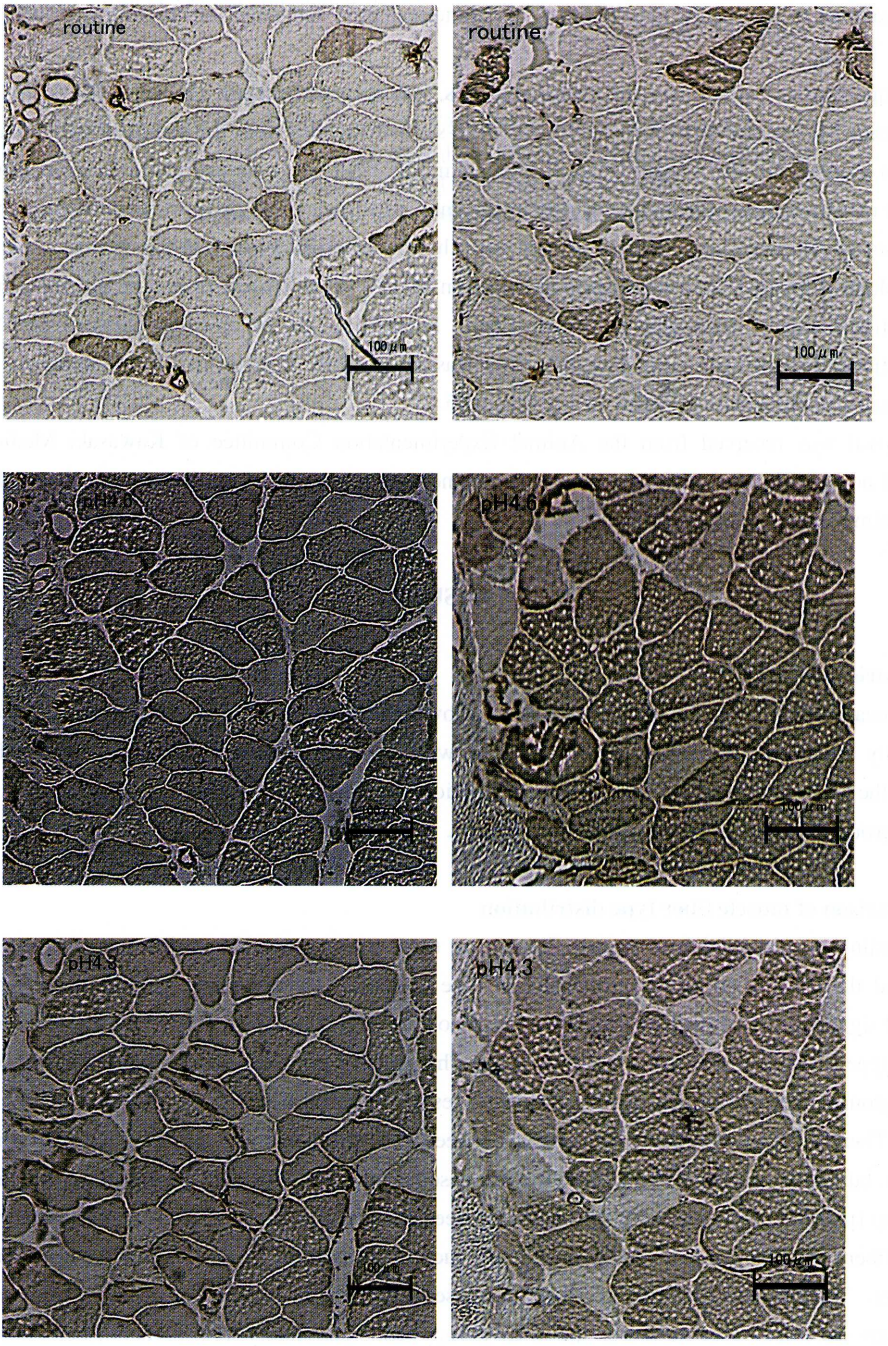


Fig 6. Typical S-MUP for MFCV measurement in the right soleus of a rat with chronic cerebral hypoperfusion.

correctly from neighboring pairs of electrodes, four waveforms were recorded at the 4-channel recorder (Fig. 6). The stimulation sites were changed repeatedly, and more than 20 new S-MUPs were detected.

MFCV was calculated based on the relationship between each interelectrode distance (2mm) and the difference in negative peak latency for four waveforms. Waveforms that showed no time lag between neighboring waves were determined to be in a motor end plate zone, and were excluded from MFCV measurements.

Following measurements of MFCV, the soleus was excised for histochemcal assessment, frozen in isopentane solution (-160°) cooled with liquid nitrogen and stored in a deep freezer (-80°). Each sample was cut into 8μm consecutive cross sections with a freezing microtome (CM3050S from Leica). ATPase staining[13] was used to distinguish, different muscle fiber types according to the classification reported by Dubowitz and Brooke[13]. That is, muscle fibers lightly stained by routine ATPase were considered to be type 1 fibers while those darkly stained were considered to be type 2 fibers; Type 2 fibers inactivated by a pretreatment solution of pH4.6 were considered to be type 2A fibers, those inactivated by a pretreatment solution of pH4.3 were considered to be type 2B fibers, and those not inactivated by solutions of either pH



(a) Ch1 **(b) Ch2**

Fig 7. Soleus muscle fibers stained by ATPase staining (×8).
Consecutive ATPase-stained sections of the soleus muscle fibers **(a)** in a rat four weeks after vascular occlusion and **(b)** in a rat eight weeks after vascular occlusion.

were considered to be type 2C muscle fibers. Room temperature and water temperature were kept constantly at 21 ± 1 ℃ (Fig. 7).

For quantitative assessment of tissue samples, microscope images (Biozero BZ-8000 All-in-one Fluorescence Microscope from Keyence) of ATPase-stained samples from the soleus of individual rats were input into a computer; the number of each type of muscle fiber was counted, and a cross-sectional area of each type of muscle fiber was measured with image analysis software (IPLab from Solution Systems).

For statistical processing, control groups and chronic cerebral hypoperfusion groups were compared using a Mann-Whitney U test with regard to the mean MFCV value, muscle fiber type distribution and mean cross-sectional area of each type of muscle fiber. In addition, the relationship between MFCV and the cross-sectional area of each type of muscle fiber was analyzed by Pearson correlation analysis. Statistical significance was defined as a p-value of less than 0.05.

Approval was received from the Animal Experimentation Committee of Kawasaki Medical School (approval no. 06-055) for all of the experiments, and they were conducted based on Kawasaki Medical School animal care and use guidelines.

RESULTS

1. Comparison of MFCV

The mean MFCV value for the Ch1 group was slower than that for the Co1 group, and the difference was statistically significant ($p<0.01$). However, there were no significant differences in mean MFCV value between the Ch2 group and the Co2 group. In addition, the mean MFCV value was significantly slower for the Ch1 group than the Ch2 group ($p<0.01$) (Table 1).

2. Comparison of muscle fiber type distribution

Regarding the distribution of muscle fiber types, there were no significant differences between the Ch1 group and the Co1 group in the proportion of type 1 fibers. However, the proportion of type 1 fibers increased significantly for the Ch2 group in comparison to the Co2 group ($p<0.01$). Although these tended to be more type 1 fibers in the Ch2 group than in the Ch1 group, there were no significant differences between the two groups. There were no significant differences in the proportion of type 2A fibers among any of the groups. There were no significant differences between Ch1 group and Co1 group in the proportion of type 2B fibers, but the proportion of type 2B fibers decreased significantly for the Ch2 group in comparison to the Co2 group ($p<0.01$). No significant difference between the Ch1 group and Ch2 group was observed. As for type 2C fibers, the Ch1 group tended to have fewer such fibers than the Co1 group, but the difference was not significant. The proportion of type 2C fibers decreased significantly for the Ch2 group in comparison to the Co2 group ($p<0.01$). Furthermore, the Ch2 group had significantly fewer such fibers than the Ch1 group($p<0.01$) (Table 2).

3. Comparison of cross-sectional areas of muscle fibers

Mean cross-sectional areas of type 1 and type 2A fibers decreased significantly for the Ch1 group in comparison to the Co1 group ($p<0.01$),but the mean cross-sectional areas of type 1 and type 2A fibers increased significantly for the Ch2 group in comparison to the Ch1 group ($p<0.01$). There were no

Table 1. Comparison of MFCV

Co1	4.45±1.32	$p < 0.01$	
Co2	3.32±0.46		
Ch1	2.19±0.19		$p < 0.01$
Ch2	3.47±0.65		

MFCV was compared with each group. Data shows mean ± S.D.(m/s).

Table 2. Comparison of distribution of muscle fiber types

	Type1		Type2A		Type2B		Type2C		
Co1	86.9±3.0		5.2±3.4		0.6±0.8		7.2±1.7		
Co2	78.1±5.4		10.7±9.4		1.4±0.7		9.8±5.8		
Ch1	87.1±8.9	$p < 0.01$	5.6±4.4	$p < 0.01$	1.6±0.9	$p < 0.01$	5.6±5.0	$p < 0.01$	$p < 0.01$
Ch2	93.7±2.8		3.9±1.9		0.8±0.9		1.5±1.1		

Type proportion was compared with each fiber type. Data shows mean ± S.D.(%)

Table 3. Comparison of cross-sectional areas of muscle fibers

	Type1		Type2A		Type2B	Type2C	
Co1	7192.6±2463.0		7023.0±1996.6		5119.3±1308.3	6124.8±1347.8	
Co2	5139.8±2118.6	$p < 0.01$	4956.4±2119.1	$p < 0.01$	4733.8±2253.0	3161.6±1712.6	$p < 0.01$
Ch1	5178.2±1670.8		4773.5±1630.9		4870.1±3087.5	3691.1±1679.1	
Ch2	6394.5±1251.4	$p < 0.01$	5285.5±1178.7	$p < 0.01$	6199.3±1101.5	4369.6±1474.6	

Muscle fiber area was compared with each fiber type. Data shows mean ± S.D.(μm2).

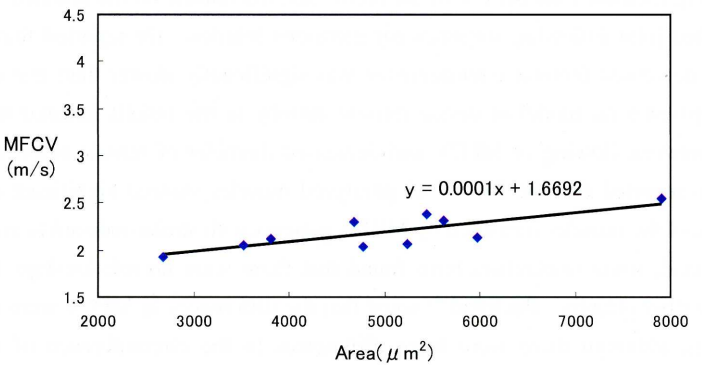


Fig 8. Relationship between MFCV and the cross-sectional area of type 1 muscle fibers in the Ch1 group (r = 0.80).

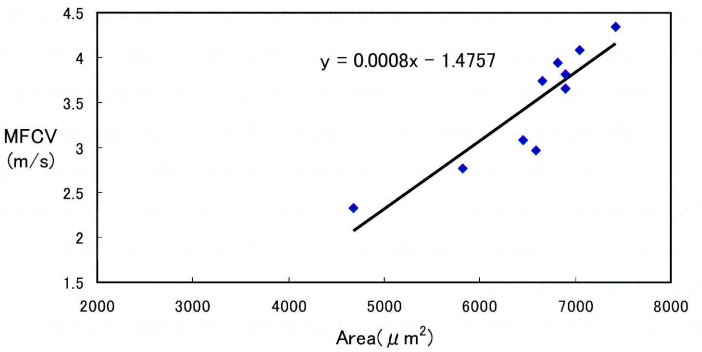


Fig 9. Relationship between MFCV and the cross-sectional area of type 1 muscle fibers in the Ch2 group (r = 0.91).

significant differences in the mean cross-sectional area of type 2B among any of the groups. The mean cross-sectional area of type 2C fibers decreased significantly for the Ch1 group as compared to that for the Co1 group ($p<0.01$). Although the mean cross-sectional area of type 2C fibers in the Ch2 group tended to

become smaller than that in the Co2 group and to become larger than that in the Ch1 group, the differences were not significant statistically (Table 3).

4. Relationship between MFCV and histochemical changes in muscle fibers

Regarding the type 1 fibers that accounted for most of the soleus muscle, there was no correlation between MFCV and the proportion of type 1 fibers. There was a significant correlation between MFCV and the cross-sectional area of type 1 fibers for both the Ch1 group (r = 0.80) and the Ch2 group (r = 0.91); the larger the muscle fiber area was, the faster the MFCV (Figs. 8, 9) was. As for type 2 fibers, there was no significant correlation between MFCV and the proportion of type 2 fibers or their cross-sectional area.

DISCUSSION

Regarding the relationship between MFCV and the size of muscle fibers, Hakansson[14] reported that there was close correlation between MFCV and circumference in the isolated single fibers of the semitendinous muscle in the frog. In addition, Stalberg[3], Troni[15], Martinez[16], and Broman[17] have shown in their human studies that there is close correlation between MFCV and the circumference of the measured body site. Specifically, Stalberg measured the MFCV of the rectus femoris muscle on the affected side in patients with an immobilized knee joint following surgeries for meniscus injuries. He reported that the MFCV on the affected side with decreased femoral circumference was significantly slower than that on the sound side[3]. Kondo *et al*[8]. prepared a rat model of disuse muscle atrophy in the tibialis anterior muscle, and found a close correlation between slowing of MFCV and decreased diameter of muscle fibers on the disused side. Kondo *et al.*[6] also reported that the MFCV of paralyzed muscles showed significant correlation with the cross-sectional area of the muscles measured by MRI in patients with stroke-induced hemiplegia.

On the other hand, some researchers have found that there were no relationships between slowing of MFCV and muscle fiber atrophy. Buchthal[18] noted that the differences in MFCV were quite small between children and adults, although there were large differences in the circumference of muscle fibers. He concluded that there were no relationships between MFCV and the size of muscle fibers. In addition, Miyata *et al*[19]. and Sadoyama *et al*[20]. measured the muscle fiber diameter of the vastus lateralis muscles biopsied from athletes following measurement of MFCV in the same muscles,and reported that MFCV did not correlate with muscle fiber diameter, but that there was a close correlation between the proportion of fast-twitch fibers (type 2 fibers) and the area they occupied. However, the measurement techniques used in all of these studies differed, and there has been little discussion about these issues. There have only a few reports regarding the relationship between MFCV and muscle fiber types. Toikawa *et al*[21]. and Shindo *et al*[22]. suggested that muscles consisting mostly of type 2 fibers had faster MFCV than those consisting mostly of type 1 fibers (slow-twitch fibers).

The surgical procedures for the chronic cerebral hypoperfusion model used in the present study were carried out in accordance with the methods of Otori *et al*[9].. Cerebral blood flow was reported to decrease about 50% over the five weeks following bilateral common carotid artery occlusion[23]. In addition, Otori reported that it decreased to 33-58% in the cerebral white matter, basal ganglia, and the cerebral cortex as a whole two days after occlusion; it improved to 51-63% in the cerebral white matter, basal ganglia, and occipital lobe after four weeks, and improved to 70-89% after eight weeks, although the blood flow did not

recover completely[9]. Based on previous reports, cerebral blood flow was undoubtedly reduced four weeks after vascular occlusion, and that the slowed MFCV and decrease in muscle fiber area could be easily accountable in this study.

In enzyme histochemical studies, Scelsi *et al*[24]., Slager *et al*[25]., Hachisuka *et al*[7]., and Dattola *et al*[26]. reported that significant type 2 fiber atrophy was found in muscles affected by central motor paralysis. Moreover, Hachisuka *et al*[7]. stated that muscular atrophy in the patients with central nervous system disorders was primarily due to disuse atrophy. However, in the present study, type 1 fibers were atrophied markedly. The difference in type atrophy might be attributable to the fact that the soleus used in this model consisted mostly of type 1 fibers. Additionally, it might be the reason that the course and period for creating disuse atrophy differed from those of other studies.

The reduced cerebral blood flow was considered to have improved eight weeks after vascular occlusion, since both the mean MFCV value and the muscle fiber area returned to levels almost equal to those in the normal rat. In this regard, observation of the rats indicated that there were no remarkable differences in movements between the Ch1 group and the Ch2 group. In terms of the distribution of muscle fiber type, however, a histochemical abnormality still remained. The proportion of type 1 fibers increased and type 2B and 2C fibers decreased significantly eight weeks after vascular occlusion in comparison to the normal rat. Tsubahara reported that progressive conversion processes of "2A → 2C → 1" and "2B → 2C → 1" seemed to occur in the soleus of sedentary rats as a result of aging[27]. The changes in muscle fiber type distribution as a result of the reduced cerebral blood flow seemed to resemble the changes caused by aging. It is suggested that the conversion of muscle fiber types takes a long time. Although cerebral blood flow recovered and the rats could exercise much, the extent of exercise might differ from that performed by the normal rats.

Since the mean MFCV value became slower and the mean cross-sectional areas of types 1, 2A, and 2C fibers decreased following reduction in the cerebral blood flow, it was suggested that MFCV changes in accordance with the size of muscle fibers. Therefore, the relationship between MFCV and the size of muscle fibers was examined, and a significant correlation between the two parameters was certified in type 1 fibers, but not in type 2 fibers. This result does not conflict with the fact that the mean MFCV value increased after the improvement of cerebral blood flow to almost the same level as that in the normal rat, because the mean cross-sectional area of the muscle fibers also increased simultaneously with that of 16-week-old normal rats. While the mean cross-sectional area of the type 1 fibers that accounted for most of the soleus showed a close correlation with the mean MFCV value, there was no significant correlation between MFCV and the cross-sectional area of type 2 fibers. If the reports[21),22)] that MFCVs recorded from type 2 fibers were faster than those from type 1 fibers are accepted, it is possible that all of the S-MUPs were recorded from type 1 fibers in my study. The decreased MFCV values returned to normal levels eight weeks after vascular occlusion, although the proportion of type 1 fibers increased and that of type 2B and 2C fibers decreased. This finding also supports the hypothesis that all of the S-MUPs might have been recorded only from type 1 fibers. For the measurement of MFCV, MPS was used as the method of nerve stimulation. To obtain each muscle action potential of a single motor unit, attention was paid to achieving the minimum stimulation intensity level at which on S-MUP could be recorded. Since it has been reported that weaker electrical current stimulates larger nerve fibers initially[28], large nerve fibers that innervate type 1 fibers may be stimulated selectively by the MPS method. Consequently, MFCV measured using MPS was suggested to

reflect in type 1 fibers. Lack of correlation between MFCV and the proportion of type 1 fibers also accords well with the idea that MFCV cannot be measured from type 2 fibers. The current study verified that MFCV was closely related to the cross-sectional area of type 1 fibers.

CONCLUSION

After a rat model of chronic cerebral hypoperfusion was prepared, measurements of MFCV and enzyme histochemical assessment using ATPase staining were performed in the soleus. The conclusions were as follow:

1. The mean cross-sectional area of muscle fibers was decreased in every fiber type except for type 2B after the ligation of bilateral common carotid arteries. The decrease in muscle fiber area was considered to be indicative of disuse muscle atrophy.
2. The mean MFCV value decreased significantly after the ligation of bilateral common carotid arteries, and there was a significant correlation between MFCV and muscle fiber area.
3. No significant correlation was found between MFCV and the distribution of muscle fiber types. Although both muscle fiber atrophy and decreased MFCV improved in eight weeks after the ligation of bilateral common carotid arteries, conversion of muscle fiber types from type 2 to type 1 progressed more in the experimental rats than in the normal rats. Muscle fiber changes resembling aging may have occurred as a result of reduced cerebral blood flow.
4. MFCV measured using MPS was suggested to reflect in type 1 fibers.

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